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(54) Title: ADENOVIRAL VECTORS FOR GENE DELIVERY IN SKELETAL MUSCLE CELLS OR MYOBLASTS

(57) Abstract: The invention provides means and methods for transduction of a skeletal muscle cell and/or a muscle cell specific precursor thereof. Provided is the use of a gene delivery vehicle derived from an adenovirus, having a tropism for said cells, for the preparation of a medicament. In a preferred aspect of the invention, said gene delivery vehicle comprises at least a tropism determining part of an adenoviral fiber protein of subgroup B and/or F. More preferably, said gene delivery vehicle comprises at least part of a fiber protein of an adenovirus of stereotype (11, 16, 35, 40 and/or 51) or a functional part, derivative and/or analogue thereof.

Title:

ADENOVIRAL VECTORS FOR GENE DELIVERY IN SKELETAL MUSCLE CELLS OR MYOBLASTS

FIELD OF THE INVENTION

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The invention relates to the field of molecular genetics and medicine. More in particular the invention relates to adenoviral vectors for targeting to skeletal muscle and/or myoblast cells.

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BACKGROUND OF THE INVENTION

The technology of delivering genetic material to
15   cells is currently wide spread and finds many applications. With the technology at hand, it is possible to transfer a wide variety of nucleic acids into a wide variety of different cell types. Some of the more classical technologies include calcium phosphate precipitation, liposome mediated
20   transfer, viral vector mediated transfer, particle bombardment and electroporation.

However, there is no ideal system for the transfer of nucleic acid into cells. All of the transfer systems of the art have their advantages and disadvantages. For instance,
25   the calcium phosphate technology has been shown to be very effective in the transfection of many *in vitro* growing immortalized cell lines, however, transfer of nucleic acid into many primary cells both *in vitro* and *in vivo* has proven to be very difficult. In fact, efficient transfer of nucleic
30   acid in primary cells has only become broadly applicable with the development of systems that use viral elements. Such viral vector systems utilize the very efficient mechanisms that viruses have developed to introduce their genetic

information into the target cell. One of the best-studied
viral vector systems is the adenovirus vector system.

Gene-transfer vectors derived from adenoviruses (so-
called adenoviral vectors) have a number of features that
5 make them particularly useful for gene transfer. 1) the
biology of the adenoviruses is characterized in detail, 2)
the adenovirus is not associated with severe human pathology,
3) the virus is extremely efficient in introducing its DNA
into the host cell, 4) the virus can infect a wide variety of
10 cells and has a broad host-range, 5) the virus can be
produced at high virus titers in large quantities, and 6) the
virus can be rendered replication defective by deletion of
the early-region 1 (E1) of the viral genome (Brody and
Crystal 1994).

15 The adenovirus genome is a linear double-stranded DNA
molecule of approximately 36000 base pairs. The adenovirus
DNA contains identical Inverted Terminal Repeats (ITR) of
approximately 90-140 base pairs with the exact length
depending on the serotype. The viral origins of replication
20 are within the ITRs exactly at the genome ends. Most
adenoviral vectors currently used in gene therapy have a
deletion in the E1 region, where novel genetic information
can be introduced. The E1 deletion renders the recombinant
virus replication defective (Levrero et al. 1991). It has
25 been demonstrated extensively that recombinant adenovirus, in
particular serotype 5, is suitable for efficient transfer of
genes *in vivo* to the liver, the airway epithelium and solid
tumors in animal models and human xenografts in immuno-
deficient mice (Bout 1996; Blaese et al. 1995). Thus,
30 preferred methods for *in vivo* gene transfer into target cells
make use of adenoviral vectors as gene delivery vehicles. At
present, six different subgroups of human adenoviruses have
been proposed which in total encompasses 51 distinct
adenovirus serotypes. Besides these human adenoviruses an

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extensive number of animal adenoviruses have been identified
(Ishibashi and Yasue 1984).

A serotype is defined on the basis of its
immunological distinctiveness as determined by quantitative
5   neutralization with animal antisera (horse, rabbit). If
neutralization shows a certain degree of cross-reaction
between two viruses, distinctiveness of serotype is assumed
if A) the hemagglutinins are unrelated, as shown by lack of
cross-reaction on hemagglutination-inhibition, or B)
10 substantial biophysical/ biochemical differences in DNA exist
(Francki et al. 1991). The nine serotypes identified last
(42-51) were isolated for the first time from HIV- infected
patients (Hierholzer et al. 1988; Schnurr and Dondero 1993;
De Jong et al. 1999). For reasons not well understood, most
15 of such immuno-compromised patients shed adenoviruses that
were rarely or never isolated from immuno-competent
individuals (Hierholzer et al. 1988; Khoo et al. 1995; De
Jong et al. 1999). The adenovirus serotype 5 (Ad5) is most
widely used for gene therapy purposes. Similar to serotypes
20 2, 4 and 7, serotype 5 has a natural affiliation towards lung
epithelia and other respiratory tissues. In contrast, it is
known that, for instance, serotypes 40 and 41 have a natural
affiliation towards the gastrointestinal tract. For a
detailed overview of the disease association of the different
25 adenovirus serotypes see table 1. In this table there is one
deviation from the literature. Sequence analysis and
hemagglutination assays using erythrocytes from different
species performed in our institute indicated that in contrast
to the literature (De Jong et al. 1999) adenovirus 50 proved
30 to be a D group vector whereas adenovirus 51 proved to be a
B-group vector. The natural affiliation of a given serotype
towards a specific organ can either be due to a difference in
the route of infection i.e. make use of different receptor
molecules or internalization pathways. However, it can also

be due to the fact that a serotype can infect many
tissues/organs but it can only replicate in one organ because
of the requirement of certain cellular factors for
replication and hence clinical disease. At present it is
5   unknown which of the above mentioned mechanisms is
responsible for the observed differences in human disease
association. However it is known that different adenovirus
serotypes can bind to different receptors due to sequence
dissimilarity of the capsid proteins i.e. hexon, penton, and
10   fiber protein. It is predominantly fiber that is responsible
for the initial attachment and thus determines the host-range
of a serotype. For instance, it has been shown that
adenoviruses of subgroup C such as Ad2 and Ad5 bind to
different receptors as compared to adenoviruses from subgroup
15   B such as Ad3 (Defer et al. 1990). Likewise, it was
demonstrated that receptor specificity could be altered by
exchanging the Ad3 with the Ad5 knob protein, and vice versa
(Krasnykh et al. 1996; Stevenson et al. 1995 and 1997).
     The initial step for successful infection is binding
20   of adenovirus to a cell surface, a process mediated through
fiber protein. The fiber protein has a trimeric structure
(Stouten et al. 1992) with different lengths, depending on
the virus serotype (Signas et al. 1985; Kidd et al. 1993).
Different serotypes have polypeptides with structurally
25   similar N and C termini, but different middle stem regions.
N-terminally, the first 30 amino acids are involved in
anchoring of the fiber to the penton base (Chroboczek et al.
1995), especially the conserved FNPVYP region in the tail
(Arnberg et al. 1997). The C-terminus, or knob, is
30   responsible for initial interaction with the cellular
adenovirus receptor. After this initial binding secondary
binding between the capsid penton base and cell-surface
integrins is proposed to lead to internalization of viral
particles in coated pits and endocytosis (Morgan et al. 1969;

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Svensson and Persson 1984; Varga et al. 1991; Greber et al.
1993; Wickham et al. 1995). Integrins are αβ-heterodimers of
which at least 14 α-subunits and 8 β-sububits have been
identified (Hynes 1992). The array of integrins expressed in
5   cells is complex and will vary between cell types and
cellular environment. Although the knob contains some
conserved regions, between serotypes, knob proteins show a
high degree of variability, indicating that different
adenovirus receptors might exist. For instance, it has been
10   demonstrated that adenoviruses of subgroup C (Ad2, Ad5) and
adenoviruses of subgroup B (Ad3) bind to different receptors
(Defer et al. 1990). Using baculovirus produced soluble CAR
as well as adenovirus serotype 5 knob protein, Roelvink et
al. (1998) concluded by using interference studies that all
15   adenovirus serotypes, except serotypes of subgroup B, enter
cells via CAR.

        Although adenovirus vectors have been used to
transfer foreign genetic material into a large variety of
cell types, skeletal muscle cells (also referred to as muscle
20   cells) and muscle cell specific precursors thereof
(myoblasts) have until the present invention at least in part
resisted efficient gene delivery by adenovirus vectors. Until
the present invention, the limited capability of adenovirus
vectors to deliver a nucleic acid of interest to said
25   (precursor) muscle cell was a problem in gene therapy.
Effective gene delivery in said cells is desirable, because
in the field of gene therapy there is an interest in skeletal
muscle as a target for treatment of many different diseases
(reviewed in DiEdwardo et al. 1999). For instance, via direct
30   injection of skeletal muscle with either recombinant viruses
(Hartigan-O'Connor and Chamberlain 2000; Akkaraju et al.
1999), naked DNA (Li S. et al. 1999) or liposome complexed
DNA, researchers are attempting to treat local muscle disease
such as Duchenne muscular dystrophy (DMD). In DMD, proper

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dystrophin protein expression is impaired due to genetic
deletions. However, other structural proteins such as
lamanin-alpha-2-chain, or delta-sarcoglyan cause other types
of muscle disease and are therefore also subject of
5   investigation (Vilquin et al. 1999).

Besides muscle diseases, a large number of other
human diseases can potentially be treated if highly efficient
transduction of skeletal muscle would occur, since skeletal
muscle presents a large mass of the human body that is easily
10   accessible. For instance, expression of proteins that are
either wrongly expressed or not expressed at all by a patient
suffering from one of many glycogen storage disorders could
potentially be treated. Genes known to be non-functional are
for instance Aldolase A, Phosphorylase B-kinase, acid
15   maltase, or ormyophosphorylase (reviewed in DiMauro and Bruno
1998).

Another example of potential areas in which efficient
genetic modification of skeletal muscle could be beneficial
are ischemia and peripheral vascular disease because
20   induction of angiogenesis has been shown to be thera-
peutically beneficial (Isner 2000, Schratzberger et al.
2000). Factors that can induce or inhibit angiogenesis are
for instance cardiotropin-1, ang1-7, NOSIII, ATF-BPTI, and
vascular endothelial growth factor (Bordet et al. 1999;
25   Rivard et al. 1999). Another example is the genetic
modification of the skeletal muscle such that it becomes a
"production factory" for therapeutic proteins that can exert
their therapeutic effect at sites distant from the skeletal
muscle (MacColl et al. 1999). Examples of the latter include
30   growth factors and hormones i.e. EPO, TGF-β, TNF-α, or IL1-
IL12 (Ueno et al. 2000; Dalle et al. 1999), blood clotting
factors i.e. factor VIII and factor IX (Chao et al. 1999)),
neurotropic factors i.e. GDNF or NGF (Mohajeri et al. 1999),
or lysosomal lipid degradation enzymes i.e. galactosidase,

glucocerebrosidase, ceraminidase. Expression of these
proteins in the muscle could be beneficial to treat for
instance anemia, blood clotting disorders, or Gaucher disease
to name but a few.

5   Because direct injection of recombinant viruses,
naked DNA or liposome complexed DNA has so far not resulted
in highly efficient genetic modification of skeletal muscle,
alternatives of direct administration approaches are
explored. In one such strategy, myoblasts cultured from adult
10   skeletal muscle biopsies are isolated. Myoblasts are
subsequently genetically modified and re-infused into the
patient, for instance in the skeletal muscle, cardiac muscle
or perhaps even in the blood stream giving the fact that
these cells might possess the ability to home to skeletal
15   muscle (Jackson et al. 1999). Transplantation into ischemic
regions of a diseased heart of genetically modified myoblasts
expressing angiogenic factors might counteract heart failure
(Scorsin et al. 2000). This strategy in which genetically
modified cells are transplanted directly is an autologous
20   procedure meaning that cells have to be derived from the
patient itself. To broaden the number of applications several
polymers have been constructed in which allogeneic cells are
encapsulated using semi-permeable membranes which thus
shields the cells from the immune system of the host (Li RH.
25   et al. 1998 and 1999). Encapsulated myoblasts thus secrete
therapeutic proteins but the myoblasts are protected from the
immune system and/or complement inactivation (Regulier et al.
1998; Dalle et al. 1999). Encapsulated cells are surgically
implanted at any easy accessible site in the body, i.e.
30   subcutaneous, intra dermal, intra peritoneal, or intra
muscular. Another alternative strategy for the direct
administration of recombinant viruses, naked DNA, or
liposome-complexed DNA is the use of myoblasts for *ex vivo*
tissue engineering also referred to as a "neo-organ

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approach". This strategy is based on the implantation of
cells, either or not genetically modified, in biodegradable
scaffolds *in vitro* (Rosenthal and Kohler 1997, Yoo and Atala
1997, Moullier et al. 1993). The cells adhere to the scaffold
5   after which the scaffold is surgically transplanted. This
approach can be used to generate bioartificial muscle (BAM)
in bioreactors (Powell et al. 1999). Genes of interest to
transfer to myoblasts using this approach are many and
include all examples listed so far. Naturally the same
10 approaches can be followed when undesired genes or proteins
have to be eliminated from the body. Hereto, for instance
anti-sense molecules (Soreq and Seidman 2000), ribozymes
(Morino et al. 2000), or chimeraplasts (Rando et al. 2000)
can be expressed in myoblasts.
15   From the above, non-limiting, examples it can be
concluded that efficient transduction of skeletal muscle
cells and myoblasts is of great importance. However, although
researchers perform a great variety of beneficial
applications with gene therapy involving skeletal muscle
20 cells and myoblasts, until the present invention there were
no suitable methods for transduction of a skeletal muscle
cell and/or a myoblast, a muscle cell specific precursor of
at least a skeletal muscle cell.
          Transduction of a (precursor) muscle cell with an
25 adenovirus vector was not efficiently possible. Until the
present invention, gene delivery was performed in a variety
of ways, which were for instance laborious, and/or
inefficient. The present invention solves the problem, how a
muscle cell and/or a specific precursor thereof can be
30 efficiently infected by a gene delivery vehicle, preferably
comprising an adenoviral vector. With the present invention
it is for instance possible to perform a wide range of
beneficial applications on a wide scale. An efficient way of
transducing said (precursor) muscle cells by an adenovirus

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vector might for instance improve the time and/or extent of treatment of a disease by gene therapy.

5   BRIEF DESCRIPTION OF THE TABLES AND FIGURES

Table 1: Association of different human adenovirus serotypes with human disease.

10   Table 2: Production results of recombinant fiber chimaeric adenoviruses. Results in virus particles per milliliter as determined by HPLC. These yields were obtained starting from 9x T175 flask giving full CPE with 2-3 days after virus inoculation.

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Figure 1: Three independent experiments (Figure 1A, 1B, and 1C respectively) were performed in which fiber chimaeric vectors were tested for their ability to transduce human A459 lung carcinoma cells. A549 cells were seeded at a density of
20   10^5 cells per well of 24-well plates and exposed twenty-four hours after seeding to an increasing dose of virus particles per cells of different fiber chimaeric vectors. The number on the X-axis represents the fiber number (i.e. 16 means Ad5Fib16). The dose of virus particles per cell used differs
25   between 1A, 1B, and 1C ranging from 10 to 5000 virus particles per cell (dose is indicated on the right). Luciferase activity is expressed in relative light units (RLU) per microgram total cellular protein.

30   Figure 2: (A) Fiber chimaeric viruses were tested for their ability to transduce mouse C2C12 myoblasts. The virus dose used of each chimaeric vector was 10, 50, 250, 1250, 2500, and 5000 virus particles per cell. Luciferase activity is expressed in relative light units (RLU) per microgram total

cellular protein. (B) Mouse C2C12 myoblasts were cultured to
high confluency such that myotube structures are
spontaneously formed. The virus dose used of each chimaeric
vector is 10, 50, 250, 1250, 2500, and 5000 virus particles
5   per cell. Luciferase activity is expressed in relative light
units (RLU) per microgram total cellular protein.

Figure 3: Fiber chimaeric viruses were tested for their
ability to transduce human primary myoblasts. The virus dose
10   used of each chimaeric fiber virus is 50 or 500 virus
particles per cell. Luciferase activity is expressed in
relative light units (RLU) per microgram total cellular
protein.

15   Figure 4: Transduction of human primary myoblasts with fiber
chimaeric vectors Ad5.Fib16, Ad5.Fib35, and Ad5Fib51 carrying
green fluorescent protein as a marker gene (GFP). Non-
transduced cells were used to set the gate on 1% GFP positive
cells. Values are corrected for background. A) Shown is the
20   percentage of GFP positive cells as determined by flow
cytometry forty-eight hours after virus exposure. B) Shown is
the median fluorescence intensity of the cells which is a
parameter that determines how much GFP is produced inside a
cell.

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Figure 5: Human *musculus pectoralis major* samples were
incubated for two hours with different fiber chimaeric
vectors at 10^{10} virus particles in 200 µl medium. Forty-eight
hours after virus exposure the samples were analyzed for
30   luciferase transgene expression. The luciferase activity
expressed in relative light units (RLU) is corrected for the
amount of protein present in the different lysates.

Figure 6: *In vivo* luciferase activity in lung and liver cells
from mice administered with Ad5 and Ad5Fib35 recombinant
adenoviruses. Shown is the average luciferase activity +/-
SD of ten mice per group. Values are corrected for negative
5 control background (n=3 mice).

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DETAILED DESCRIPTION

        The present invention provides means and methods for
the efficient transduction of a skeletal muscle cell and/or a
5   muscle cell specific precursor thereof. In one aspect the
     invention discloses a gene delivery vehicle for delivering a
     nucleic acid of interest to a skeletal muscle cell and/or a
     myoblast cell, comprising a recombinant adenovirus having a
     tropism for skeletal muscle cells and/or myoblast cells and a
10   nucleic acid encoding at least one amino acid sequence that
     is able to counteract a cardiovascular disease and/or a
     disease which affects skeletal muscle cells and/or myoblasts.
     In one embodiment, said disease that affects skeletal muscle
     cells and/or myoblasts is Duchenne muscular dystrophin and/or
15   a glycogen storage disorder. Now that efficient transduction
     of a muscle cell and/or myoblast by a gene delivery vehicle
     is disclosed, it is possible to at least in part improve the
     efficacy of medical applications.
        For efficient transduction of skeletal muscle cells
20   and/or myoblasts, adenoviruses are favored because of the
     high levels of expression. However, although transduction of
     a skeletal muscle cell and/or a muscle cell specific
     precursor thereof is possible with adenovirus serotype 5, Ad5
     efficiently infects liver cells, lung epithelia and other
25   respiratory tissues. This is a problem, because if Ad5 is
     used to transduce a certain nucleic acid sequence of interest
     to a skeletal muscle cell and/or myoblast, said nucleic acid
     of interest can also be expressed by liver cells and/or cells
     of the respiratory tract. Expression of said nucleic acid of
30   interest in liver cells and/or cells of the respiratory tract
     is not desirable, because this may cause many side-effects,
     like lysis of said cells or lysis of cells surrounding an
     infected cell, depending on the transgene. This might occur
     for instance when dead-proteins or other apoptosis-inducing

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·genes are being used. ·Another non-desired side-effect might
be the triggering of immune responses towards virus-infected
cells that are not present in the tissue that is being
treated. Thus, preferably, a gene delivery vehicle of the
5   invention does not infect many liver cells and/or many
respiratory cells. Besides, transduction of a skeletal muscle
cell and/or a muscle cell specific precursor thereof by
adenovirus serotype 5 is not very efficient. Therefore, high·
titers of Ad5 are needed. This has similar disadvantages, for
10   instance toxicity to cells and for instance a strong immune
response.

     The present invention discloses a gene delivery
vehicle with a tropism for a skeletal muscle cell and/or a
myoblast cell. Said skeletal muscle cell and/or myoblast cell
15   may be a primary skeletal muscle cell and/or a primary
myoblast cell. In one embodiment, the invention discloses a
gene delivery vehicle, comprising a recombinant adenovirus,
wherein said recombinant adenovirus is a chimaeric
adenovirus.

20   A chimaeric adenovirus comprises a chimaeric adenovirus
capsid or equivalent thereof. A chimaeric adenovirus capsid
comprises at least a functional part of a capsid protein that
is derived from one adenovirus serotype and at least a
functional part of another capsid protein that is derived
25   from another adenovirus serotype.

     In a preferred embodiment said chimaeric capsid
comprises a fiber protein comprising a tropism determining
part from an adenovirus serotype belonging to group B or
group F, and a penton/hexon anchoring part derived from a
30   fiber protein of adenovirus serotype 5. Said chimaeric capsid
further comprises hexon and penton proteins derived from
adenovirus serotype 5.

     Another aspect of the present invention discloses a
gene delivery vehicle comprising a recombinant adenovirus,

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wherein said recombinant adenovirus comprises at least a
tropism determining part of an adenoviral fiber protein
comprising a tropism for skeletal muscle cells and/or
myoblast cells. Said tropism may be provided by at least a
5   tropism determining part of an adenoviral fiber protein of
subgroup B and/or F. Preferably, a gene delivery vehicle of
the invention comprises at least part of a fiber protein of
an adenovirus of serotype 11, 16, 35, 40 and/or serotype 51
or a functional derivative and/or analogue of said serotype.
10   In one embodiment, a gene delivery vehicle of the
invention comprising an adenoviral vector comprises a
deletion in the gene encoding for fiber protein. Said
deletion may be replaced, on that site or on a different site
of said vector, by a nucleic acid sequence encoding an amino
15 acid sequence capable of conferring onto said gene delivery
vehicle a tropism for a skeletal muscle cell and/or a
myoblast. Said nucleic acid sequence preferably comprises at
least a functional part of the fiber protein from an
adenovirus from subgroup B or F, comprising at least the
20 binding moiety of the fiber protein. More preferably, a gene
delivery vehicle of the invention comprises at least part of
a fiber protein of an adenovirus of serotype 11, 16, 35, 40,
and/or serotype 51 or a functional part, derivative and/or
analogue thereof.
25   A gene delivery vehicle of the invention comprising
at least part of the short fiber protein 40-S can be very
well used for transduction of a human skeletal muscle cell.
A gene delivery vehicle of the invention comprising at least
part of the long fiber protein 40-L can be used for
30 transduction of a rodent myoblast.
To reduce a strong immune response of the host, the
invention also discloses a gene delivery vehicle, preferably
comprising at least a functional part of the fiber protein
from an adenovirus from subgroup B and/or F, more preferably

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comprising at least a functional part of a fiber protein of
an adenovirus of serotype 11, 16, 35, 40 and/or serotype 51
or a functional derivative and/or analogue thereof, which
comprises hexon and/or penton, or a functional part,
derivative and/or analogue thereof, which is not derived from
an adenovirus of serotype 5. Preferably, a gene delivery
vehicle of the invention comprises a capsid derived from an
adenovirus of subgroup B. More preferably, said capsid is
derived from an adenovirus of serotype 11, 26 and/or 35, or a
functional part, derivative and/or analogue thereof. Capsids
of serotypes 11, 26, 35 and the natural occuring 11/35
chimaeric vector are less immunogenic in a human body.

A gene delivery vehicle of the invention may at least
in part reduce a strong immune response, because if Ad5 is
used to transduce skeletal muscle cells and/or muscle cell
specific precursors thereof, an immunocompetent host will
produce antibodies against Ad5. A gene delivery vehicle of
the invention can be used to transduce skeletal muscle cells
and/or primary myoblasts, either after a former treatment
with Ad5 or instead of a treatment with Ad5. The host may
already contain antibodies against the Ad5 hexon and/or
penton. But these antibodies are less, if at all, able to
bind hexon and/or penton of a gene delivery vehicle of the
invention, which preferably comprises hexon and/or penton
from an adenovirus of serotype 11, 26 and/or 35, or a
functional part, derivative and/or analogue thereof. When a
gene delivery vehicle of the invention is administered, the
host will start producing antibodies against the new hexon
and/or penton. This will take a certain amount of time. Thus,
at least in the beginning of a treatment with a gene delivery
vehicle of the invention, immune response may be less than it
would be if Ad5 were used. Once a host has produced
antibodies against a certain gene delivery vehicle of the
invention, another gene delivery vehicle of the invention,

comprising another hexon and/or penton or a functional part, derivative and/or analogue thereof, can be used to at least partly reduce the immune response again.

As used herein, a gene delivery vehicle is a carrier
5   which is able to deliver at least one nucleic acid to a host cell. If a gene delivery vehicle comprises a tropism for a certain kind of host cell, said gene delivery vehicle comprises at least a high affinity to bind to said host cell. Said gene delivery vehicle may, or may not, bind to other
10   kind of cells as well, with either the same or a different affinity. Preferably, a gene delivery vehicle of the invention has a higher affinity to skeletal muscle cells and/or myoblasts, and/or a lower affinity to liver cells, lung epithelia and other respiratory tissues, than an
15   adenovirus serotype 5 wild type. Use of a gene delivery vehicle of the invention for transduction of skeletal muscle cells and/or myoblasts with high efficacy, causes less toxic side-effects than would be the case if Ad5 were used, because in the present invention less virus can be used to transduce
20   the same amount of cells.

A functional part of a molecule is a part of a molecule that comprises the same kind of properties of said molecule in kind, not necessarily in amount. A non-limiting example of a functional part of a fiber protein is a knob
25   protein. A functional derivative of a molecule is a molecule that has been altered such that the properties of said molecule are essentially the same in kind, not necessarily in amount. A derivative can be provided in many ways. A derivative of a protein can for instance be provided through
30   conservative amino acid substitution. A person skilled in the art is well able to generate analogous compounds of, for instance, a fiber protein and/or hexon and/or penton. This can for instance be done through screening of a peptide library. Such an analogue has essentially the same properties

of the fiber protein and/or hexon and/or penton in kind, not necessarily in amount.

A gene delivery vehicle of the invention preferably comprises a vector derived from subgroup B or subgroup F

5   adenoviruses. More preferably, said vector is derived from serotype 11, 16, 35, 40 and/or 51. After a host cell is infected by an adenovirus, a nucleic acid of interest that is delivered does not integrate into the genome of the host cell. Therefore, it is surprising that an adenoviral vector

10   can be used for transduction of myoblasts, because if a myoblast is infected by an adenoviral vector, a nucleic acid of interest which is delivered to said myoblast is very likely to be lost in progeny muscle cells. Therefore, for a myoblast, one would be tempted to choose a method of gene

15   delivery whereby a nucleic acid of interest is known to become part of the genome of the host cell. However, using a gene delivery vehicle of the invention we have now found that adenovirus can also be used. The high efficacy of a gene delivery vehicle of the invention apparently enables

20   sufficient nucleic acid to enter the cells such that upon division of a myoblast cell, at least part of the progeny also comprises said nucleic acid. Thus, the present invention discloses a method of transducing a myoblast by a gene delivery vehicle of the invention. For many applications

25   transient expression in myoblasts of exogenous genes appears to be sufficient to trigger a process, for example angiogenesis. For several other applications sustained expression is necessary, for instance when myoblasts are encapsulated in artificial polymers to allow secretion of

30   amino acid sequences, according to the neo-organ-approach. For all applications, a gene delivery vehicle of the invention can be used, since an adenovirus can be engineered to integrate into the host cell genome (Conçalves et al. 2000). A gene delivery vehicle of the invention is also

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suitable for delivering a nucleic acid of interest to a
skeletal muscle cell, because non-genomic expression is
sufficient in this case. Thus, in one embodiment the
invention provides a use of a gene delivery of the invention,
5   comprising a tropism for skeletal muscle cells and/or
myoblast cells, as a vehicle for delivering a nucleic acid of
interest to a skeletal muscle cell and/or a myoblast cell.
Said skeletal muscle cell and/or myoblast cell may be a
primary cell.

10   In another embodiment the present invention
provides methods and means by which chimaeric adenoviruses
based on adenovirus serotype 5 with modified fiber genes can
be generated. For this purpose, two or three plasmids, which
together contain the complete adenovirus serotype 5 genome,
15   may be constructed. From this plasmid the DNA encoding the
adenovirus serotype 5 fiber protein may be removed and
replaced by linker DNA sequences that facilitate easy
cloning. A plasmid in which the native adenovirus serotype 5
fiber sequence is partially removed may subsequently serve as
20   a template for insertion of DNA encoding for fiber protein
derived from different adenovirus serotypes (human or
animal). The DNAs derived from different serotypes may be
obtained using the polymerase chain reaction technique in
combination with (degenerated) oligo-nucleotides. At the
25   former E1 location in the genome of adenovirus serotype 5,
any gene of interest can be cloned. A single transfection
procedure of the two or three plasmids together may result in
the formation of a recombinant chimaeric adenovirus. Although
successful introduction of changes in the adenovirus serotype
30   5 fiber and penton-base have been reported by others, the
complex structure of knob and the limited knowledge of the
precise aminoacids interacting with CAR render such targeting
approaches laborious and difficult. To overcome the
limitations described above, pre-existing adenovirus fibers

may be used to maximize the chance of obtaining recombinant
adenovirus which can normally assemble in the nucleus of a
producer cell and which can be produced on pre-existing
packaging cells.

5   In another aspect the invention provides a use of a
gene delivery vehicle of the invention for the preparation of
a medicament. Of course, a gene delivery vehicle of the
invention can also be used for prophylactic purposes.
Therefore, in yet another aspect, the invention provides a
10   use of a gene delivery vehicle of the invention for the
preparation of a vaccine. Said gene delivery vehicle may be
used for the delivery of a nucleic acid of interest to a
muscle cell and/or a myoblast. Therefore, this aspect of the
invention is of course particularly useful for the treatment
15   of diseases which affect muscle cells and/or muscle cell
specific precursors thereof, for instance Duchenne muscular
dystrophin, or glycogen storage disorders. Said aspect of the
invention is also very useful for the treatment of a
cardiovascular disease. A gene delivery vehicle of the
20   invention is able to deliver a nucleic acid sequence
efficiently. Therefore it makes a major contribution to less
efficient treatments of diseases that affect muscle cells.
           Alternatively, this aspect of the invention is also
useful for expression of potential therapeutic genes in the
25   muscle of which the amino acid sequence can (also) exert its
action elsewhere. Thus, yet another embodiment of the
invention discloses a use of a skeletal muscle cell and/or
myoblast cell comprising at least one therapeutic gene,
provided by a gene delivery vehicle comprising a recombinant
30   adenovirus having a tropism for skeletal muscle cells and/or
myoblast cells, for expression of a therapeutic amino acid
sequence which can exert its action inside and/or outside
said skeletal muscle cell and/or myoblast cell.

In yet another aspect of the invention a nucleic acid
is disclosed, which encodes at least part of a gene delivery
vehicle of the invention. Of course, expression of a nucleic
acid of the invention is particularly useful to generate a
5   gene delivery vehicle of the invention. Expression may be
accomplished in any way known in the art.

In yet another aspect, the invention discloses an
isolated cell, said cell comprising a gene delivery vehicle
of the invention. Said cell may comprise a nucleic acid as
10   described in the previous paragraph. Thus, another embodiment
of the invention discloses an isolated cell, said cell
comprising a nucleic acid that encodes at least part of a
gene delivery vehicle of the invention.

Typically, one does not want an adenovirus batch to
15   be administered to a host cell that contains replication
competent adenovirus, although this is not always true. In
general therefore it is desired to omit a number of genes
(but at least one) from the adenoviral genome on the vector
encoding the recombinant virus and to supply these genes in
20   a cell in which the vector is brought to produce recombinant
adenovirus. Such a cell is usually called a packaging cell.

A gene delivery vehicle of the invention preferably
comprises deletions in the E1, E2, E3 and/or E4 region. In
that case, proteins that normally are encoded in said
25   regions are not expressed in a gene delivery vehicle of the
invention. To generate a new gene delivery vehicle of the
invention, said protein or proteins must be produced
separately. Therefore, in one embodiment the invention
discloses a cell of the invention, further comprising a
30   nucleic acid encoding an adenovirus early protein or a
functional part, derivative and/or analogue thereof.
Preferably said adenovirus early protein comprises an E1,
E2, E3 and/or E4 region encoded protein. A nucleic acid
encoding said adenovirus early protein might be located in

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the genome of a cell of the invention, although other
locations are possible. Said adenovirus early protein may be
derived from an adenovirus of subgroup B or subgroup F. Thus
in another embodiment the invention discloses a cell of the
5   invention, comprising an adenovirus early protein of an
adenovirus of subgroup B or subgroup F. Typically vector and
packaging cell have to be adapted to one another in that
they have all the necessary elements, but that they do not
have overlapping elements which lead to replication
10   competent virus by recombination. Of course, a gene of
interest can be inserted at for instance the site of E1 of
the original adenovirus from which the vector is derived. In
this manner the chimaeric adenovirus to be produced can be
adapted to the requirements and needs of certain hosts in
15   need of gene therapy for certain disorders.
        A cell of the invention as described in the preceding
paragraph is of course particularly well suitable for the
production of a gene delivery vehicle of the invention.
Therefore, in another aspect the invention provides use of a
20   cell of the invention for production of a gene delivery
vehicle of the invention. A product of a cell of the
invention may enable the preparation of a pharmaceutical.
Thus, another aspect of the invention is use of a cell of the
invention for the preparation of a pharmaceutical. In one
25   embodiment the invention provides a use of a cell of the
invention for the preparation of a vaccine.
        Another aspect of the invention discloses a skeletal
muscle cell and/or myoblast, provided with an additional
nucleic acid encoding at least one amino acid sequence that
30   is able to counteract a disease that affects muscle cells
and/or myoblasts. Said disease may be Duchenne muscular
dystrophin and/or a glycogen storage disorder. Also, said
disease may be a cardiovascular disease.

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In another aspect, the invention discloses a skeletal
muscle cell and/or myoblast provided with at least one
potential therapeutic gene of which the amino acid sequence
can exert its action inside and/or outside said skeletal
5   muscle cell and/or myoblast cell.
A skeletal muscle cell and/or myoblast cell of the invention
may be a primary skeletal muscle cell and/or a primary
myoblast cell. Another embodiment of the invention discloses
a pharmaceutical composition comprising a gene delivery
10   vehicle of the invention.

EXAMPLES

The examples below illustrate the present invention.
They are not limiting the invention in any way. With the
5 teaching of the present invention, a person skilled in the
art can perform alternative experiments that are still in
the scope of the present invention.

Example 1: Generation of adenovirus serotype 5 genomic
10 **plasmid clones**

The complete genome of adenovirus serotype 5 has been
cloned into various plasmids or cosmids to allow easy
modification of parts of the adenovirus serotype 5 genome
retaining the capability to produce recombinant virus. For
15· this purpose the following plasmids were generated:

1. pBr/Ad.Bam-rITR (ECACC deposit P97082122)
In order to facilitate blunt end cloning of the ITR
sequences, wild-type human adenovirus type 5 (Ad5) DNA was
20 treated with Klenow enzyme in the presence of excess dNTPs.
After inactivation of the Klenow enzyme and purification by
phenol/chloroform extraction followed by ethanol
precipitation, the DNA was digested with BamHI. This DNA
preparation was used without further purification in a
25 ligation reaction with pBr322 derived vector DNA prepared as
follows: pBr322 DNA was digested with EcoRV and BamHI,
dephosphorylated by treatment with TSAP enzyme (Life
Technologies) and purified on LMP agarose gel (SeaPlaque
GTG). After transformation into competent *E.coli* DH5α (Life
30 Techn.) and analysis of ampiciline resistant colonies, one
clone was selected that showed a digestion pattern as ·
expected for an insert extending from the BamHI site in Ad5
to the right ITR. Sequence analysis of the cloning border at
the right ITR revealed that the most 3′ G residue of the ITR

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was missing, the remainder of the ITR was found to be
correct. Said missing G residue is complemented by the other
ITR during replication of the virus in packaging cells.

5   2. pBr/Ad.Sal-rITR (ECACC deposit P97082119)
pBr/Ad.Bam-rITR was digested with BamHI and SalI. The vector
fragment including the adenovirus insert was isolated in LMP
agarose (SeaPlaque GTG) and ligated to a 4.8 kb SalI-BamHI
fragment obtained from wt Ad5 DNA and purified with the
10   Geneclean II kit (Bio 101, Inc.). One clone was chosen and
the integrity of the Ad5 sequences was determined by
restriction enzyme analysis. Clone pBr/Ad.Sal-rITR contains
adeno type 5 sequences from the SalI site at bp 16746 up to
and including the rITR (missing the most 3' G residue).
15

3. pBr/Ad.Cla-Bam (ECACC deposit P97082117)
Wild type Adeno type 5 DNA was digested with ClaI and BamHI,
and the 20.6 kb fragment was isolated from gel by electro-
elution. pBr322 was digested with the same enzymes and
20   purified from agarose gel by Geneclean. Both fragments were
ligated and transformed into competent DH5α. The resulting
clone pBr/Ad.Cla-Bam was analysed by restriction enzyme
digestion and shown to contain an insert with adenovirus
sequences from bp 919 to 21566.
25

4. pBr/Ad.AflII-Bam (ECACC deposit P97082114)
Clone pBr/Ad.Cla-Bam was linearized with EcoRI (in pBr322)
and partially digested with AflII. After heat inactivation of
AflII for 20' at 65°C the fragment ends were filled in with
30   Klenow enzyme. The DNA was then ligated to a blunt double
stranded oligo linker containing a PacI site (as
underlined)(5'-AATTGTCTTAATTAACCGCTTAA-3'). This linker was
made by annealing the following two oligonucleotides: 5'-
AATTGTCTTAATTAACCGC-3' and 5'-AATTGCGGTTAATTAAGAC-3',

25

followed by blunting with Klenow enzyme. After precipitation
of the ligated DNA to change buffer, the ligations were
digested with an excess PacI enzyme to remove concatameres of
the oligo. The 22016 bp partial fragment containing Ad5

5   sequences from bp 3534 up to 21566 and the vector sequences,
was isolated in LMP agarose (SeaPlaque GTG), religated and
transformed into competent DH5α. One clone that was found to
contain the PacI site and that had retained the large adeno
fragment was selected and sequenced at the 5' end to verify

10   correct insertion of the PacI linker in the (lost) AflII
site.

5. pBr/Ad.Bam-rITRpac#2 (ECACC deposit P97082120) and
pBr/Ad.Bam-rITR#8 (ECACC deposit P97082121)

15   To allow insertion of a PacI site near the ITR of Ad5 in
clone pBr/Ad.Bam-rITR about 190 nucleotides were removed
between the ClaI site in the pBr322 backbone and the start of
the ITR sequences. This was done as follows: pBr/Ad.Bam-rITR
was digested with ClaI and treated with nuclease Bal31 for

20   varying lengths of time (2', 5', 10' and 15'). The extent of
nucleotide removal was followed by separate reactions on
pBr322 DNA (also digested at the ClaI site), using identical
buffers and conditions. Bal31 enzyme was inactivated by
incubation at 75°C for 10 min, the DNA was precipitated and

25   resuspended in a smaller volume of TE buffer. To ensure blunt
ends, DNAs were further treated with T4 DNA polymerase in the
presence of excess dNTPs. After digestion of the (control)
pBr322 DNA with SalI, satisfactory degradation (~150 bp) was
observed in the samples treated for 10' or 15'. The 10' or

30   15' treated pBr/Ad.Bam-rITR samples were then ligated to the
above described blunted PacI linkers (See pBr/Ad.AflII-Bam).
Ligations were purified by precipitation, digested with
excess PacI and separated from the linkers on an LMP agarose
gel. After religation, DNAs were transformed into competent

DH5α and colonies analyzed. Ten clones were selected that
showed a deletion of approximately the desired length and
these were further analyzed by T-track sequencing (T7
sequencing kit, Pharmacia Biotech). Two clones were found
5   with the PacI linker inserted just downstream of the rITR.
After digestion with PacI, clone #2 has 28 bp and clone #8
has 27 bp attached to the ITR.

pWE/Ad.AflII-rITR (ECACC deposit P97082116)
10 Cosmid vector pWE15 (Clontech) was used to clone larger Ad5
inserts. First, a linker containing a unique PacI site was
inserted in the EcoRI sites of pWE15 creating pWE.pac. To
this end, the double stranded PacI oligo as described for
pBr/Ad.AflII-BamHI was used but now with its EcoRI protruding
15 ends. The following fragments were then isolated by electro-
elution from agarose gel: pWE.pac digested with PacI,
pBr/AflII-Bam digested with PacI and BamHI and pBr/Ad.Bam-
rITR#2 digested with BamHI and PacI. These fragments were
ligated together and packaged using 1 phage packaging
20 extracts (Stratagene) according to the manufacturer's
protocol. After infection into host bacteria, colonies were
grown on plates and analyzed for presence of the complete
insert. pWE/Ad.AflII-rITR contains all adenovirus type 5
sequences from bp 3534 (AflII site) up to and including the
25 right ITR (missing the most 3' G residue).

pBr/Ad.lITR-Sal(9.4) (ECACC deposit P97082115)
Ad5 wild type DNA was treated with Klenow enzyme in the
presence of excess dNTPs and subsequently digested with SalI.
30 Two of the resulting fragments, designated left ITR-Sal(9.4)
and Sal(16.7)-right ITR, respectively, were isolated in LMP
agarose (Seaplaque GTG). pBr322 DNA was digested with EcoRV
and SalI and treated with phosphatase (Life Technologies).
The vector fragment was isolated using the Geneclean method

(BIO 101, Inc.) and ligated to the Ad5 SalI fragments. Only
the ligation with the 9.4 kb fragment gave colonies with an
insert. After analysis and sequencing of the cloning border a
clone was chosen that contained the full ITR sequence and
5 extended to the SalI site at bp 9462.

pBr/Ad.1ITR-Sal(16.7) (ECACC deposit P97082118)
pBr/Ad.1ITR-Sal(9.4) is digested with SalI and de-
phosphorylated (TSAP, Life Technologies). To extend this
10 clone upto the third SalI site in Ad5, pBr/Ad.Cla-Bam was
linearized with BamHI and partially digested with SalI. A 7.3
kb SalI fragment containing adenovirus sequences from 9462-
16746 was isolated in LMP agarose gel and ligated to the
SalI-digested pBr/Ad.1ITR-Sal(9.4) vector fragment.
15

pWE/Ad.AflII-EcoRI
pWE.pac was digested with ClaI and 5' protruding ends were
filled using Klenow enzyme. The DNA was then digested with
PacI and isolated from agarose gel. pWE/AflII-rITR was
20 digested with EcoRI and after treatment with Klenow enzyme
digested with PacI. The large 24 kb fragment containing the
adenoviral sequences was isolated from agarose gel and
ligated to the ClaI-digested and blunted pWE.pac vector using
the Ligation Express™ kit (Clontech). After transformation
25 of Ultracompetent XL10-Gold cells (Stratagene), clones were
identified that contained the expected insert. pWE/AflII-
EcoRI containes Ad5 sequences from bp 3534-27336.

Construction of new adapter plasmids
30 The absence of sequence overlap between the recombinant
adenovirus and E1 sequences in the packaging cell line is
essential for safe, RCA-free generation and propagation of
new recombinant viruses. The adapter plasmid pMLPI.TK is an
example of an adapter plasmid designed for use according to

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the invention in combination with the improved packaging cell
lines of the invention. This plasmid was used as the starting
material to make a new vector in which nucleic acid molecules
comprising specific promoter and gene sequences can be easily
5   exchanged. First, a PCR fragment was generated from
pZipΔMo+PyF101(N⁻) template DNA (described in WO 96/35798)
with the following primers: LTR-1: 5′-CTG TAC GTA CCA GTG CAC
TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3′ and LTR-2: 5′-GCG
GAT CCT TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG
10   GCG ACT CAG TCA ATC G-3′. Pwo DNA polymerase (Boehringer
Mannheim) was used according to manufacturers' protocol with
the following temperature cycles: once 5′ at 95°C; 3′ at
55°C; and 1′ at 72°C, and 30 cycles of 1′ at 95°C, 1′ at
60°C, 1′ at 72°C, followed by once 10′ at 72°C. The PCR
15   product was then digested with BamHI and ligated into pMLP10
(Levrero et al. 1991) vector digested with PvuII and BamHI,
thereby generating vector pLTR10. This vector contains
adenoviral sequences from bp 1 up to bp 454 followed by a
promoter consisting of a part of the Mo-MuLV LTR having its
20   wild-type enhancer sequences replaced by the enhancer from a
mutant polyoma virus (PyF101). The promoter fragment was
designated L420. Next, the coding region of the murine HSA
gene was inserted. pLTR10 was digested with BstBI followed by
Klenow treatment and digestion with NcoI. The HSA gene was
25   obtained by PCR amplification on pUC18-HSA (Kay et al. 1990)
using the following primers: HSA1, 5′-GCG CCA CCA TGG GCA GAG
CGA TGG TGG C-3′ and HSA2, 5′-GTT AGA TCT AAG CTT GTC GAC ATC
GAT CTA CTA ACA GTA GAG ATG TAG AA-3′. The amplified fragment
(269 bp) was subcloned in a shuttle vector using the NcoI and
30   BglII sites. Sequencing confirmed incorporation of the
correct coding sequence of the HSA gene, but with an extra
TAG insertion directly following the TAG stop codon. The
coding region of the HSA gene, including the TAG duplication
was then excised as a NcoI (sticky)-SalI (blunt) fragment and

cloned into the 3.5 kb NcoI(sticky)/BstBI(blunt) fragment
from pLTR10, resulting in pLTR-HSA10. Finally, pLTR-HSA10 was
digested with EcoRI and BamHI after which the fragment
containing the left ITR, packaging signal, L420 promoter and

5   HSA gene was inserted into vector pMLPI.TK digested with the
same enzymes and thereby replacing the promoter and gene
sequences. This resulted in the new adapter plasmid pAd/L420-
HSA that contains convenient recognition sites for various
restriction enzymes around the promoter and gene sequences.

10   SnaBI and AvrII can be combined with HpaI, NheI, KpnI,
HindIII to exchange promoter sequences, while the latter
sites can be combined with the ClaI or BamHI sites 3' from
HSA coding region to replace genes in this construct.
Another adapter plasmid that was designed to allow easy

15   exchange of nucleic acid molecules was made by replacing the
promoter, gene and poly (A) sequences in pAd/L420-HSA with
the CMV promoter, a multiple cloning site, an intron and a
poly-A signal. For this purpose, pAd/L420-HSA was digested
with AvrII and BglII followed by treatment with Klenow to

20   obtain blunt ends. The 5.1 kb fragment with pBr322 vector and
adenoviral sequences was isolated and ligated to a blunt 1570
bp fragment from pcDNA1/amp (Invitrogen) obtained by
digestion with HhaI and AvrII followed by treatment with T4
DNA polymerase. This adapter plasmid was named pCLIP.

25

Generation of recombinant adenoviruses
To generate E1 deleted recombinant adenoviruses with the new
plasmid-based system, the following constructs are prepared:
a) An adapter construct containing the expression cassette

30   with the gene of interest linearized with a restriction
enzyme that cuts at the 3' side of the overlapping adenoviral
genome fragment, preferably not containing any pBr322 vector
sequences, and b) A complementing adenoviral genome construct
pWE/Ad.AflII-rITR digested with PacI. These two DNA molecules

are further purified by phenol/ chloroform extraction and
EtOH precipitation. Co-transfection of these plasmids into an
adenovirus packaging cell line, preferably a cell line
according to the invention, generates recombinant replication

5   deficient adenoviruses by a one-step homologous recombination
between the adapter and the complementing construct.
Alternatively, instead of pWE/Ad.AflII-rITR other fragments
can be used, e.g., pBr/Ad.Cla-Bam digested with EcoRI and
BamHI or pBr/Ad.AflII-BamHI digested with PacI and BamHI can

10   be combined with pBr/Ad.Sal-rITR digested with SalI. In this
case, three plasmids are combined and two homologous
recombination events are needed to obtain a recombinant
adenovirus. It is to be understood that those skilled in the
art may use other combinations of adapter and complementing

15   plasmids without departing from the present invention.
A general protocol as outlined below and meant as a non-
limiting example of the present invention has been performed
to produce several recombinant adenoviruses using various
adapter plasmids and the Ad.AflII-rITR fragment. Adenovirus

20   packaging cells (PER.C6™, ECACC deposit 96022940) were
seeded in ~25 cm² flasks and the next day when they were at
~80% confluency, transfected with a mixture of DNA and
lipofectamine agent (Life Techn.) as described by the
manufacturer. Routinely, 40 µl lipofectamine, 4 µg adapter

25   plasmid and 4 µg of the complementing adenovirus genome
fragment AflII- rITR (or 2 µg of all three plasmids for the
double homologous recombination) are used. Under these
conditions transient transfection efficiencies of ~50% (48
hrs post transfection) are obtained as determined with

30   control transfections using a pAd/CMV-LacZ adapter. Two days
later, cells are passaged to ~80 cm² flasks and further
cultured. Approximately five (for the single homologous
recombination) to eleven days (for the double homologous
recombination) later a cytopathogenic effect (CPE) is seen,

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indicating that functional adenovirus has formed. Cells and
medium are harvested upon full CPE and recombinant virus is
released by freeze/thawing. An extra amplification step in an
80 cm² flask is routinely performed to increase the yield

5   since at the initial stage the titers are found to be
variable despite the occurrence of full CPE. After
amplification, viruses are harvested and plaque purified on
PER.C6™ cells. Individual plaques are tested for viruses
with active transgenes. Besides replacements in the E1 region

10   it is possible to delete or replace (part of) the E3 region
in the adenovirus because E3 functions are not necessary for
the replication, packaging and infection of the (recombinant)
virus. This creates the opportunity to use a larger insert or
to insert more than one gene without exceeding the maximum

15   package size (approximately 105% of wt genome length). This
can be done, e.g., by deleting part of the E3 region in the
pBr/Ad.Bam-rITR clone by digestion with XbaI and religation.
This removes Ad5 wt sequences 28592-30470 including all known
E3 coding regions. Another example is the precise replacement

20   of the coding region of gp19K in the E3 region with a
polylinker allowing insertion of new sequences. This, 1)
leaves all other coding regions intact and 2) obviates the
need for a heterologous promoter since the transgene is
driven by the E3 promoter and poly (A) sequences, leaving

25   more space for coding sequences. To this end, the 2.7kb EcoRI
fragment from wt Ad5 containing the 5' part of the E3 region
was cloned into the EcoRI site of pBluescript (KS⁻)
(Stratagene). Next, the HindIII site in the polylinker was
removed by digestion with EcoRV and HincII and subsequent

30   religation. The resulting clone pBS.Eco-Eco/ad5DHIII was used
to delete the gp19K coding region. Primers 1 (5'-GGG TAT TAG
GCC AA AGG CGC A-3') and 2 (5'-GAT CCC ATG GAA GCT TGG GTG
GCG ACC CCA GCG-3') were used to amplify a sequence from
pBS.Eco-Eco/Ad5DHIII corresponding to sequences 28511 to

28734 in wt Ad5 DNA. Primers 3 (5'-GAT CCC ATG GGG ATC CTT
TAC TAA GTT ACA AAG CTA-3') and 4 (5'-GTC GCT GTA GTT GGA CTG
G-3') were used on the same DNA to amplify Ad5 sequences from
29217 to 29476. The two resulting PCR fragments were ligated
5   together by virtue of the new introduced NcoI site and
subsequently digested with XbaI and MunI. This fragment was
then ligated into the pBS.Eco-Eco/ad5ΔHIII vector that was
digested with XbaI (partially) and MunI generating pBS.Eco-
Eco/ad5ΔHIII.Δgp19K. To allow insertion of foreign genes into
10   the HindIII and BamHI site, an XbaI deletion was made in
pBS.Eco-Eco/ad5ΔHIII.Δgp19K to remove the BamHI site in the
Bluescript polylinker. The resulting plasmid pBS.Eco-
Eco/ad5ΔHIIIΔgp19KΔXbaI, contains unique HindIII and BamHI
sites corresponding to sequences 28733 (HindIII) and 29218
15   (BamHI) in Ad5. After introduction of a foreign gene into
these sites, either the deleted XbaI fragment is re-
introduced, or the insert is recloned into pBS.Eco-Eco/ad5Δ
HIII.Δgp19K using HindIII and for example MunI. Using this
procedure, we have generated plasmids expressing HSV-TK,
20   human IL-1a, rat IL-3, luciferase or LacZ. The unique SrfI
and NotI sites in the pBS.Eco-Eco/ad5ΔHIII.Δ gp19K plasmid
(with or without inserted gene of interest) are used to
transfer the region comprising the gene of interest into the
corresponding region of pBr/Ad.Bam-rITR, yielding construct
25   pBr/Ad.Bam-rITRΔgp19K (with or without inserted gene of
interest). This construct is used as described *supra* to
produce recombinant adenoviruses. In the viral context,
expression of inserted genes is driven by the adenovirus E3
promoter. Recombinant viruses that are both E1 and E3 deleted
30   are generated by a double homologous recombination procedure
as described above for E1-replacement vectors using a
plasmid-based system consisting of: a) an adapter plasmid for
E1 replacement according to the invention, with or without

insertion of a first gene of interest, b) the pWE/Ad.AflII-
EcoRI fragment, and c) the pBr/Ad.Bam-rITRΔgp19K plasmid with
or without insertion of a second gene of interest. In
addition to manipulations in the E3 region, changes of (parts
of) the E4 region can be accomplished easily in pBr/Ad.Bam-
rITR. Generation and propagation of such a virus, however, in
some cases demands complementation *in trans*.

**Example 2: Generation of adenovirus serotype 5 based viruses
with chimaeric fiber proteins**

The method described *infra* to generate recombinant
adenoviruses by co-transfection of two, or more seperate
cloned adenovirus sequences. One of these cloned adenovirus
sequences was modified such that the adenovirus serotype 5
fiber DNA was deleted and substituted for unique restriction
sites thereby generating "template clones" which allow for
the easy introduction of DNA sequences encoding for fiber
protein derived from other adenovirus serotypes.

Generation of adenovirus template clones lacking DNA encoding
for fiber

The fiber coding sequence of adenovirus serotype 5 is located
between nucleotides 31042 and 32787. To remove the adenovirus
serotype 5 DNA encoding fiber we started with construct
pBr/Ad.Bam-rITR. First a NdeI site was removed from this
construct. For this purpose, pBr322 plasmid DNA was digested
with NdeI after which protruding ends were filled using
Klenow enzym. This pBr322 plasmid was then re-ligated,
digested with NdeI and transformed into *E.coli* DH5α. The
obtained pBr/ΔNdeI plasmid was digested with ScaI and SalI
and the resulting 3198 bp vector fragment was ligated to the
15349 bp ScaI-SalI fragment derived from pBr/Ad.BamrITR,
resulting in plasmid pBr/Ad.Bam-rITRΔNdeI which hence
contained a unique NdeI site. Next a PCR was performed with

oligonucleotides NY-up: 5'- CGA **CAT ATG** TAG <u>ATG CAT</u> TAG TTT
GTG TTA TGT TTC AAC GTG-3' and NY-down: 5'-GGA GAC CAC TGC
CAT GTT-3'. During amplification, both a NdeI (bold face) and
a NsiI restriction site (underlined) were introduced to

5   facilitate cloning of the amplified fiber DNAs. Amplification
consisted of 25 cycles of each 45 sec. at 94°C, 1 min. at
60°C, and 45 sec. at 72°C. The PCR reaction contained 25 pmol
of oligonucleotides NY-up or NY-down, 2 mM dNTP, PCR buffer
with 1.5 mM MgCl₂, and 1 unit of Elongase heat stable

10   polymerase (Gibco, The Netherlands). 10% of the PCR product
was run on an agarose gel that demonstrated that the expected
DNA fragment of ± 2200 bp was amplified. This PCR fragment
was subsequently purified using Geneclean kit system (Bio101
Inc.). Then, both the construct pBr/Ad.Bam-rITRΔNdeI as well

15   as the PCR product was digested with restriction enzymes NdeI
and SbfI. The PCR fragment was subsequently cloned using T4
ligase enzyme into the NdeI and SbfI digested pBr/Ad.Bam-rITR
ΔNdeI, generating pBr/Ad.BamRΔFib. This plasmid allows
insertion of any PCR amplified fiber sequence through the

20   unique NdeI and NsiI sites that are inserted in place of the
removed fiber sequence. Viruses can be generated by a double
homologous recombination in packaging cells described *infra*
using an adapter plasmid, construct pBr/Ad.AflII-EcoRI
digested with PacI and EcoRI and a pBr/Ad.BamRΔFib construct

25   in which heterologous fiber sequences have been inserted. To
increase the efficiency of virus generation, the construct
pBr/Ad.BamRΔFib was modified to generate a PacI site flanking
the right ITR. Hereto, pBr/Ad.BamRΔFib was digested with
AvrII and the 5 kb adenovirus fragment was isolated and

30   introduced into the vector pBr/Ad.Bam-rITR.pac#8 replacing
the corresponding AvrII fragment. The resulting construct was
named pBr/Ad.BamRΔFib.pac. Once a heterologous fiber sequence
is introduced in pBr/Ad.BamRΔFib.pac, the fiber modified

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right hand adenovirus clone may be introduced into a large
cosmid clone as described for pWE/Ad.AflII-rITR in example 1.
Such a large cosmid clone allows generation of adenovirus by
only one homologous recombination making the process

5   extremely efficient.

<u>Amplification of fiber sequences from adenovirus serotypes</u>
To enable amplification of the DNAs encoding fiber protein
derived from alternative serotypes degenerate oligo-

10   nucleotides were synthesized. For this purpose, first known
DNA sequences encoding for fiber protein of alternative
serotypes were aligned to identify conserved regions in both
the tail-region as well as the knob-region of the fiber
protein. From the alignment, which contained the nucleotide

15   sequence of 19 different serotypes representing all 6
subgroups, (degenerate) oligonucleotides were synthesized
(table 2). Also depicted in table 2 is the combination of
oligonucleotides used to amplify DNA encoding fiber protein
of a specific serotype. The amplification reaction (50 µl)

20   contained 2 mM dNTPs, 25 pmol of each oligonucleotide,
standard 1x PCR buffer, 1,5 mM MgCl$_2$, and 1 Unit Pwo heat
stable polymerase (Boehringer) per reaction. The cycler
program contained 20 cycles, each consisting of 30 sec. 94°C,
60 sec. 60-64°C, and 120 sec. At 72°C. 10% of the PCR product

25   was run on an agarose gel which demonstrated that a DNA
fragment was amplified. Of each different template, two
independent PCR reactions were performed after which the
independent PCR fragments obtained were sequenced to
determine the nucleotide sequence. From 11 different

30   serotypes, the nucleotide sequence could be compared to
sequences present in genbank. Of all other serotypes, the DNA
encoding fiber protein was previously unknown and was
therefore aligned with known sequences from other subgroup
members to determine homology i.e. sequence divergence. Of

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the 51 human serotypes known to date, we have determined the
DNA sequence encoding for fiber, except for serotypes 1, 6,
18, and 26.

5 . Generation of fiber chimaeric adenoviral DNA constructs
All amplified fiber DNAs as well as the vector (pBr/Ad.BamRΔ
Fib) were digested with NdeI and NsiI. The digested DNAs was
subsequently run on an agarose gel after which the fragments
were isolated from the gel and purified using the Geneclean
10 kit (Bio101 Inc). The PCR fragments were then cloned into the
NdeI and NsiI sites of pBr/AdBamRΔFib, thus generating
pBr/AdBamRFibXX (where XX stands for the serotype number of
which the fiber DNA was isolated). So far the fiber sequence
of serotypes 5/ 7/ 8/ 9/ 10/ 11/ 12/ 13/ 14/ 16/ 17/ 19/ 21/
15 24/ 27/ 28/ 29/ 30/ 32/ 33/ 34/ 35/ 36/ 37/ 38/ 40-S/ 40-L/
41-S/ 42/45/ 47/ 49/ 51 have been cloned in pBr/AdBamRFibXX.
From pBr/AdBamRFibXX (where XX is 5/ 8/ 9/ 10/ 11/ 13/ 16/
17/ 24/ 27/ 30/ 32/ 33/ 34/ 35/ 38/ 40-S/ 40-L/ 45/ 47/ 49/
51) a cosmid clone in pWE/Ad.AflII-rITR (see example 1) was
20 generated to facilitate efficient virus generation. This
cosmid cloning resulted in the formation of construct
pWE/Ad.AflII-rITR/FibXX (where XX stands for the serotype
number of which the fiber DNA was isolated).

25 Generation of recombinant adenovirus chimaeric for fiber
protein
To generate recombinant Ad 5 virus carrying the fiber of
serotype 12, 16, 28, 40-L, 51, and 5, three constructs,
pCLIP/luciferase, pWE/AdAflII-Eco and pBr/AdBamrITR.pac/fibXX
30 were transfected into adenovirus producer cells. To generate
recombinant Ad5 virus carrying the fiber of 5/ 7/ 8/ 9/ 10/
11/ 12/ 13/ 14/ 16/ 17/ 19/ 21/ 24/ 27/ 28/ 29/ 30/ 32/ 33/
34/ 35/ 36/ 37/ 38/ 40-S/ 40-L/ 41-S/ 42/45/ 47/ 49/ 51, two

contructs pCLIP/ luciferase and pWE/Ad.AflII-rITR/FibXX were
transfected into adenovirus producer cells. For transfection,
2 μg of pCLIP/luciferase, and 4 μg of both pWE/AdAflII-Eco
and pBr/AdBamrITR.pac/fibXX (or in case of cosmids: 4 μg of
5   pCLIP/luciferase plus 4 μg of pWE/Ad.AflII-rITR/FibXX) were
diluted in serum free DMEM to 100 μl total volume. To this
DNA suspension 100 μl 1x diluted lipofectamine (Gibco) was
added. After 30 minutes at room temperature the DNA-
lipofectamine complex solution was added to 2.5 ml of serum-
10 free DMEM that was subsequently added to a T25 cm^2 tissue
culture flask. This flask contained $2x10^6$ PER.C6™ cells that
were seeded 24-hours prior to transfection. Two hours later,
the DNA-lipofectamine complex containing medium was diluted
once by the addition of 2.5 ml DMEM supplemented with 20%
15 fetal calf serum. Again 24 hours later the medium was
replaced by fresh DMEM supplemented with 10% fetal calf
serum. Cells were cultured for 6-8 days, harvested, and
freeze/thawed 3 times. Cellular debris was removed by
centrifugation for 5 minutes at 3000rpm at room temperature.
20 Of the supernatant 3-5 ml was used to again infect PER.C6
cells (T80 cm^2 tissue culture flasks). This re-infection
resulted in full cytopathogenic effect (CPE) after 5-6 days
after which the adenovirus is harvested as described above.

25 **Example 3: Production, purification, and titration of fiber
chimaeric adenoviruses**

        Of the supernatant obtained from transfected PER.C6™
cells 10 ml was used to inoculate nine T175 flasks containing
$1.5 x 10^6$ cells/ ml PER.C6™. Three days after inoculation,
30 the cells were trypsinised and pelleted by centrifugating for
10 min at 1750 rpm at room temperature. The chimaeric
adenovirus present in the pelleted cells was subsequently
extracted and purified using the following downstream

processing protocol. The pellet was dissolved in 50 ml 10 mM
NaPO$_4^-$ and frozen at -20°C. After thawing at 37°C, 5.6 ml
deoxycholate (5% w/v) was added and the solution was
homogenized. This solution was subsequently incubated for 15
min at 37°C to completely crack the cells. After homogenizing
the solution, 1875 µl (1M) MgCl$_2^-$ was added and 5 ml 100%
glycerol. After the addition of 375 µl DNAse (10 mg/ ml) the
solution was incubated for 30 min at 37°C. Cell membranes
were removed by centrifugation at 1880xg for 30 min at room
temperature without the brake on. The supernatant was
subsequently purified from proteins by loading on 10 ml of
freon. Upon centrifugation for 15 min at 2000 rpm without
brake at room temperature three bands are usually visible of
which the upper band represents the adenovirus. This band was
picked after which it was loaded on a Tris/HCl (1M) buffered
CsCl blockgradient (range: 1.2 to 1.4 gr./ml). Upon
centrifugation at 21000 rpm for 2.5 h at 10°C the virus was
purified since it does not migrate into the 1.4 g/ml CsCl
solution. The virus band was isolated after which a second
purification using a Tris/HCl (1 M) buffered continues
gradient of 1.33 g/ml of CsCl was performed. The virus was
centrifuged for 17 h at 55000 rpm at 10°C. Subsequently the
virus band was isolated and after the addition of 30 µl of
sucrose (50 w/v) excess CsCl was removed by three rounds of
dialysis, each round comprising of 1 h. For dialysis the
virus was transferred to dialysis slides (Slide-a-lizer, cut
off 10000 kDa, Pierce, USA). The buffers used for dialysis
are PBS which are supplemented with an increasing
concentration of sucrose (round 1 to 3: 30 ml, 60 ml, and 150
ml sucrose (50% w/v)/ 1.5 liter PBS, all supplemented with
7.5 ml 2% (w/v) CaMgCl$_2$). After dialysis, the virus was
removed from the slide-a-lizer after which it was stored in
portions of 25 and 100 µl at -85°C. To determine the number

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of virus particles per milliliter, 100 µl of the virus batch
was run on a high-pressure liquid chromatograph (HPLC). The
adenovirus binds to the column (anion exchange) after which
it is eluted using a NaCl gradient (range 300-600 mM). By
5   determining the area under the virus peak the number of virus
particles can be calculated. To determine the number of
infectious units (IU) per ml present in a virus batch,
titrations are performed on 911 cells. For this purpose,
$4x10^4$ 911 cells are seeded per well of 96-well plates in rows
10   B, D, and F in a total volume of 100 µl per well. Three hours
after seeding the cells are attached to the plastic support
after which the medium can be removed. To the cells a volume
of 200 µl is added, in duplicate, containing different
dilutions of virus (range: 10^2 times diluted to $2x10^9$). By
15   screening for CPE the highest virus dilution which still
renders CPE after 14 days is considered to contain at least
one infectious unit. Using this observation, together with
the calculated amount of virus volume present in these wells
renders the number of infectious units per ml of a given
20   virus batch. The production result i.e. virus particles per
ml and IU per ml of those chimaeric adenoviruses that were
produced, all with the luciferase cDNA as a marker, are shown
in table 2.

25   **Example 4: Adenovirus transduction of murine myoblasts**

       To test the viability of all fiber chimaeric vectors
generated, human lung carcinoma cells (A549, ATCC) that are
readily infectible with Ad5 were transduced with Ad5-based
vectors carrying fiber molecules derived from alternative
30   serotypes. Forty-eight hours after the addition of virus,
cells were washed twice with 1 ml PBS after which cells were
lysed by adding 100 µl of cell lysisbuffer. Lysates were
subsequently transferred to 96-well plates and stored at -
20°C. Luciferase activity was determined using a bio-

luminescence machine, the luciferase assay (Promega™ E-1501)
and the instructions provided by the manufacturer. The
results shown in figure 1 demonstrate that a clear virus dose
dependent increase in luciferase activity can be obtained on
5 A549 cells with all vectors generated except Ad5fib12 and
Ad5Fib49. Also, it becomes clear that, based on the transgene
expression levels measured, the efficiency of transduction
differs between fiber chimaeric vectors. Next, murine C2C12
myoblasts (ATCC) which were routinely maintained in
10 Dulbecco's modified Eagles medium (DMEM) supplemented with
10% fetal calf serum were tested. In a first experiment, 10^5
C2C12 cells were seeded in wells of 24-well plates. The next
day cells were exposed to an increasing amount of virus
particles per cell (10, 50 250, 1250, 2500, 5000) of
15 recombinant fiber chimaeric viruses. The viruses selected for
testing represent five of the six different subgroups: A
(Ad5Fib12), B (Ad5Fib16 and Ad5Fib51), D (Ad5Fib28, Ad5Fib32,
and Ad5Fib49) and F (Ad5Fib40-L). In this experiment, the
parental Ad5 adenoviral vector was taken along as a reference
20 and as a member of subgroup C. The results of the luciferase
transgene expression measured in mouse C2C12 cells after
transduction with the panel of fiber chimaeric viruses is
shown in figure 2a. The result shows that, of the panel
tested, one of the fiber chimaeric viruses (Ad5.Fib40-L)
25 perform better on C2C12 cells as compared to Ad5 yielding
approximately 2-3 fold higher levels of transgene expression.
In a next experiment, the same panel of fiber chimaeric
viruses was tested on C2C12 cells that were cultured to high
confluency such that the cells spontaneously form myotube
30 structures. Myotubes are differentiated C2C12 cells that fuse
together thus forming micro fibrils, the building blocks of
skeletal muscle. This experiment on myotubes was thus
performed since it better predicts the ability of the viruses
to transduce mouse skeletal muscle. For this experiment C2C12

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cells were again seeded at 10^5 in wells of 24-well plates and cultured for several days until myotube structures were clearly visible. The cells of one well were counted and based on these cell counts virus particles were added to the other wells at increasing dose (1250, 2500, 5000 virus particles per cell). Forty-eight hours later cells were harvested and subjected to lysis and luciferase measurements as described above. The result of this experiment is shown in figure 2b. The data are in agreement with the first experiment in that Ad5.Fib40-L is superior for the transduction of murine myoblasts/ myotubes as compared to the Ad5 parent vector. Interestingly the level of luciferase activity measured was much higher in myotubes than on myoblasts indicative for a better transduction efficiency overall of myotubes. Clearly, these results demonstrate that the library of fiber chimaeric vectors can also be used to identify improved adenoviral vectors for the transduction of human myoblasts.

Example 5: Adenovirus transduction of human myoblasts

To investigate whether improved vectors for genetic modification of human myoblasts are present in the library of fiber chimaeric vectors, experiments as described under example 4 were performed on human primary myoblasts. These cells were obtained from human skeletal muscle biopsies using essentially the "explant" technique described for smooth muscle cell and endothelial cell isolation from vessels (Jaffe et al. 1973; Quax et al. 1998; Wijnberg et al. 1997). Also, human primary myoblasts were obtained commercially. (Promocell™). To test the fiber chimaeric vectors, primary myoblasts were seeded at 10^5 cells per well of 24-well plates. Forty-eight hours after seeding, the cells were exposed to the library of fiber chimaeric vectors for 2 h. Then the virus was discarded and fresh medium was added to the cells. Forty-eight hours after virus exposure cells were

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harvested and luciferase activity was determined in the cell
lysate as described in example 4. The results of this
experiment are shown in figure 3. Based on the luciferase
transgene expresion several fiber chimaeric vectors show

5   increased levels of transgene expression as compared to Ad5.
Fiber chimaeric vectors that are superior to Ad5 for the
transduction of human primary myoblasts are one subgroup D
member, Ad5.Fib13 (5.7x more transgene expression and
interestingly, all vectors carrying a fiber derived from

10   subgroup B members: Ad5Fib11 (4.3x), Ad5Fib16 (6.2x),
Ad5Fib35 (6.8x), and Ad5Fib51 (12.7x). In contrast to
subgroup B, only Ad5Fib13 of the subgroup D fiber chimaeric
vectors proved superior to Ad5. All other subgroup D fiber
chimaeric vectors (Ad5.Fib10, Ad5.Fib24, Ad5.Fib27,

15   Ad5.Fib30, Ad5.Fib33, Ad5.Fib45, Ad5.Fib47) did not perform
better as compared to Ad5. In contrast to mouse C2C12 cells
subgroup F derived fiber chimaeric vectors (Ad5.Fib40-L, and
Ad5.Fib40-S) demonstrated only marginal higher luciferase
transgene activity levels compared to Ad5. The discrepancy

20   between the result obtained on mouse and human muscle cells
can be due to the fact that receptors for these different
fibers are unknown and therefore it is conceivable that
receptor molecules are not conserved among species.
To confirm the results obtained with the fiber chimaeric

25   vectors carrying luciferase an identical experiment was
performed as described above with a few selected fiber
chimaeric adenoviruses carrying green fluorescent protein
(GFP) as a marker. This marker allows single cell analysis
using a flow cytometer and thus provides additional

30   information regarding the number of cells transduced. Also,
the expression level of green fluorescent protein per cell
can be determined and is expressed in the median fluorescence
signal intensity. Since the level of expression in a cell
correlates with the number of adenoviral genomes per cell, a

high level of GFP expression correlates with more successful
infections as compared to a low level of GFP expression in a
cell. The result of this experiment is shown in figure 4a
(percentage of cells positive for GFP) and figure 4b (median

5   fluoresce intensity signal). These results confirm earlier
results in that fiber chimaeric viruses carrying the fiber
from adenovirus subgroup B members (Ad5.Fib16, Ad5.Fib35, and
Ad5.Fib51) are superior for the genetic modification of human
myoblasts as compared to Ad5. The increase in the percentage

10   of transgene positive cells as compared to Ad5 was 10.2%
(Ad5Fib16), 4.1% (Ad5Fib35), and 10.3% (Ad5Fib51). Besides
transducing a higher number of cells the median fluorescence
signal intensity (figure 4b) indicates that per cell more
viruses entered leading to higher levels of GFP per cell when

15   using fiber chimaeric vectors carrying subgroup B fiber
molecules. Because of the compact nature of skeletal muscle,
transduction of myoblasts itself might not be sufficient to
predict improved genetic modification after injection of an
adenovirus into the skeletal muscle. Therefore, in a next

20   experiment the library of fiber chimaeric vectors was tested
on skeletal muscle tissue samples. Hereto, human skeletal
muscle samples were obtained approximately 12 hours post-
mortem after informed consent of the family. Part of the
musculus pectoralis major was obtained of which 20 samples,

25   each approximately 3x3x3 mm were generated. The samples were
transferred to wells of 24-well plates in DMEM supplemented
with 10% fetal calf serum. Each sample was exposed for two
hours to one of the fiber chimaeric variants carrying
luciferase (10^{10} virus particles in 200 µl total medium).

30   After two hours virus was discarded by washing after which
the muscle samples were returned to the incubator in 1 ml of
DMEM. After 48 h samples were minced and luciferase activity
was determined in total protein lysates. The result of this
experiment is shown in figure 5. From these experiments

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several observations are made. 1) In general, fiber chimaeric
vectors that performed better (in terms of transgene
expression) than Ad5 on cultured myoblasts also perform
better on skeletal muscle biopsies. 2) On skeletal muscle
5.   tissue, Ad5Fib40-S is superior to Ad5 (3428 times increased
luciferase activity) which was not observed on cultured
myoblasts. 3) All fiber chimaeric vectors carrying the fiber
derived from subgroup B viruses are better suited to
transduce skeletal muscle as compared to Ad5. The observed
10   increase in luciferase activity was 153x (Ad5Fib11), 868x
(Ad5Fib16), 26x (Ad5.Fib35), and 1944x(Ad5.Fib51). 4)
Several, but not all, chimaeric vectors carrying a fiber from
subgroup D members yielded increased levels of luciferase
activity. These vectors included Ad5Fib13 (495x), Ad5Fib17
15   (161x), Ad5Fib38 (659x), Ad5Fib47 (22x). In contrast
Ad5Fib10, Ad5Fib24, Ad5Fib30, Ad5Fib32, and Ad5Fib45 (all
subgroup D fibers) were not superior compared to Ad5 for the
genetic modification of human skeletal muscle. The mechanism
as to why these particular fibers allow Ad5 to enter skeletal
20   muscle tissue more efficiently is unknown at present. For
many of the human adenovirus serotypes the receptor(s) are
currently unknown. One hypothesis for the better entry is
that all the identified fiber molecules contain a relatively
short shaft, thereby positioning the knob of the fiber close
25   to the adenovirus core. The diameter of Ad5 is approximately
100 nm from which the fiber protrudes another 70 nm. In total
the diameter of Ad5 is thus maximally close to 240 nm
although the fiber itself is a flexible structure. With the
short shaft the size of the fiber chimaeric vectors is
30   reduced approximately 70 nm that might be sufficient to
obtain an improved penetration into the skeletal muscle
tissue.

Example 6: Infection of liver and lung cells in rodents using Ad5 and Ad5Fib35 viruses

To investigate whether a recombinant Ad5 adenoviral
vector carrying a fiber derived from serotype 35 had a
5   different infectability as compared to recombinant Ad5 with a
natural capsid, the following experiment was performed. Ten
BalB/C mice were administered with 10^{10} virus particles of
recombinant Ad5 carrying a luciferase transgene and ten
BalB/C mice were administered with 10^{10} virus particles of a
10   luciferase carrying recombinant Ad5Fib35 batch. All mice were
injected through the tail vein. 48 hours after administration
the mice were sacrificed and the liver and lung cells were
subsequently analyzed for luciferase activity as described in
example 4. Figure 6 shows that the Ad5Fib35 exhibits a
15   significant lower luciferase activity in both liver and lung
cells as compared to the recombinant Ad5 virus that contains
an Ad5 fiber protein in its capsid. This suggests that the
Ad35 fiber protein causes a decreased affinity for liver (3-
log difference) and lung cells (2-log difference) when it is
20   incorporated into the Ad5 viral particle.

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Table 1

Syndrom	Subgenus	Serotype
Respiratory illness	A	31
	B	3, 7, 11, 14, 21, 34, 35, 51
	C	1, 2, 5, 6
	D	39, 42-48
	E	4
Keratoconjunctivitis (eye)	B	11
	D	8, 19, 37, 50
Hemorrhagic cystitis (Kidney)	B	7, 11, 14, 16, 21, 34, 35
And urogenital tract infections	C	5
	D	39, 42-48
Sexual transmission	C	2
	D	19, 37
Gastroenteritis	A	31
	B	3
	C	1, 2, 5
	D	28
	F	40, 41
CNS disease	A	12, 31
	B	3, 7
	C	2, 5, 6
	D	32, 49
Hepatitis	A	31
	C	1, 2, 5
Disseminated	A	31
	B	3, 7, 11, 21
	D	30, 43-47
None (???)	A	18
	D	9, 10, 13, 15 17, 20, 22-29, 33, 36, 38

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Table 2

Adenovirus	Virus particles/ ml
Ad5Fib5	2.2×10^{12}
Ad5Fib9	4.9×10^{11}
Ad5Fib10	5.5×10^{11}
Ad5Fib11	1.1×10^{12}
Ad5Fib12	4.4×10^{12}
Ad5Fib13	1.1×10^{12}
Ad5Fib16	1.4×10^{12}
Ad5Fib17	9.3×10^{11}
Ad5Fib24	1.0×10^{12}
Ad5Fib27	3.0×10^{11}
Ad5Fib30	7.1×10^{11}
Ad5Fib32	2.0×10^{12}
Ad5Fib33	1.5×10^{12}
Ad5Fib35	2.0×10^{12}
Ad5Fib38	5.8×10^{11}
Ad5Fib40-S	3.2×10^{10}
Ad5Fib40-L	2.0×10^{12}
Ad5Fib45	2.8×10^{12}
Ad5Fib47	2.6×10^{12}
Ad5Fib49	1.2×10^{12}
Ad5Fib51	5.1×10^{12}

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Claims

1. Use of a gene delivery vehicle having a tropism for
   skeletal muscle cells and/or myoblast cells as a vehicle
   for delivering a nucleic acid of interest to a skeletal
   muscle cell and/or myoblast cell.

2. Use according to claim 1, wherein said skeletal muscle cell
   and/or myoblast cell is a primary cell.

3. Use according to claim 1 or 2, wherein said gene delivery
   vehicle comprises a recombinant adenovirus.

4. Use according to any one of claims 1-3, wherein said gene
   delivery vehicle comprises at least a tropism determining
   part of an adenoviral fiber protein having a tropism for
   skeletal muscle cells and/or myoblast cells.

5. Use according to any one of claims 1-4, wherein said
   tropism is provided by at least a tropism determining part
   of an adenoviral fiber protein of subgroup B and/or F.

6. Use according to claim 5, wherein said fiber protein is
   derived from an adenovirus of serotype 11, 16, 35, 40
   and/or serotype 51 or a functional part, derivative and/or
   analogue of said serotype.

7. Use according to any one of claims 1-6, wherein said gene
   delivery vehicle comprises a capsid derived from an
   adenovirus of subgroup B.

8. Use according to claim 7, wherein said capsid is derived
   from an adenovirus of serotype 11, 26 and/or 35, or a
   functional part, derivative and/or analogue thereof.

9. A gene delivery vehicle for delivering a nucleic acid of
   interest to a skeletal muscle cell and/or a myoblast cell,
   comprising a recombinant adenovirus having a tropism for
   skeletal muscle cells and/or myoblast cells and a nucleic
   acid encoding at least one amino acid sequence that is able
   to counteract a cardiovascular disease and/or a disease
   which affects skeletal muscle cells and/or myoblasts.

10. A gene delivery vehicle according to claim 9, wherein said disease that affects skeletal muscle cells and/or myoblasts is Duchenne muscular dystrophin and/or a glycogen storage disorder.

11. A gene delivery vehicle according to claim 9 or 10, wherein said skeletal muscle cell and/or myoblast cell is a primary cell.

12. A gene delivery vehicle according to any one of claims 9-11, wherein said recombinant adenovirus is a chimaeric adenovirus.

13. A gene delivery vehicle according to claim 12, comprising at least a tropism determining part of an adenoviral fiber protein comprising a tropism for skeletal muscle cells and/or myoblast cells.

14. A gene delivery vehicle according to any one of claims 9-13, wherein said tropism is provided by at least a tropism determining part of an adenoviral fiber protein of subgroup B and/or F.

15. A gene delivery vehicle according to any one of claims 9-14, comprising at least part of a fiber protein of an adenovirus of serotype 11, 16, 35, 40 and/or serotype 51 or a functional derivative and/or analogue of said serotype.

16. A gene delivery vehicle according to claim 15, comprising a capsid derived from an adenovirus of subgroup B.

17. A gene delivery vehicle according to claim 15 or 16, wherein said capsid is derived from an adenovirus of serotype 11, 26 and/or 35, or a functional part, derivative and/or analogue thereof.

18. Use of a gene delivery vehicle according to any one of claims 9-17, for the preparation of a medicament.

19. Use of a gene delivery vehicle according to any one of claims 9-17, for the preparation of a vaccine.

20. Use according to claim 18 for the treatment of a disease which affects skeletal muscle cells and/or myoblast cells.

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21. Use according to claim 20, wherein said disease is
    Duchenne muscular dystrophin and/or a glycogen storage
    disorder.

22. Use according to claim 20, for the treatment of a
5   cardiovascular disease.

23. Use of a skeletal muscle cell and/or myoblast cell
    comprising at least one therapeutic gene, provided by a
    gene delivery vehicle comprising a recombinant adenovirus
    having a tropism for skeletal muscle cells and/or myoblast
10 cells, for expression of a therapeutic amino acid sequence
    which can exert its action inside and/or outside said
    skeletal muscle cell and/or myoblast cell.

24. A nucleic acid encoding a gene delivery vehicle according
    to any one of claims 9-17.

15 25. An isolated cell comprising a gene delivery vehicle
    according to any one of claims 9-17.

26. An isolated cell comprising a nucleic acid according to
    claim 24.

27. A cell according to claim 25 or 26, further comprising a
20 nucleic acid encoding an adenovirus early protein or a
    functional part, derivative and/or analogue of said
    adenovirus early protein.

28. A cell according to claim 27, wherein said adenovirus
    early protein comprises an E1, E2, E3 and/or E4 region
25 encoded protein.

29. A cell according to claim 27 or 28, wherein said cell
    comprises said nucleic acid encoding said adenovirus early
    protein, in the genome of said cell.

30. A cell according to any one of claims 25-30, comprising an
30 adenovirus early protein of an adenovirus of subgroup B or
    subgroup F.

31. Use of a cell according to any one of claims 25-30 for the
    production of a gene delivery vehicle according to any one
    of claims 9-17.

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32. Use of a cell according to any one of claims 25-30 for the
    preparation of a pharmaceutical.

33. Use of a cell according to any one of claims 25-30 for the
    preparation of a vaccine.

5   34. A skeletal muscle cell and/or myoblast cell provided with
    a nucleic acid encoding at least one amino acid sequence
    that is able to at least in part counteract a disease which
    affects muscle cells and/or myoblast cells.

35. A skeletal muscle cell and/or myoblast cell according to

10 claim 34, wherein said disease is Duchenne muscular
    dystrophin and/or a glycogen storage disorder.

36. A skeletal muscle cell and/or myoblast cell provided with
    a nucleic acid encoding at least one amino acid sequence
    that is able to at least in part counteract a

15 cardiovascular disease.

37. A skeletal muscle cell and/or myoblast cell provided with
    a therapeutic gene of which the amino acid sequence can
    exert its action inside and/or outside said skeletal muscle
    cell and/or myoblast cell.

20 38. A skeletal muscle cell and/or myoblast cell according to
    any one of claims 34-37, which is a primary cell.

39. A pharmaceutical composition comprising a gene delivery
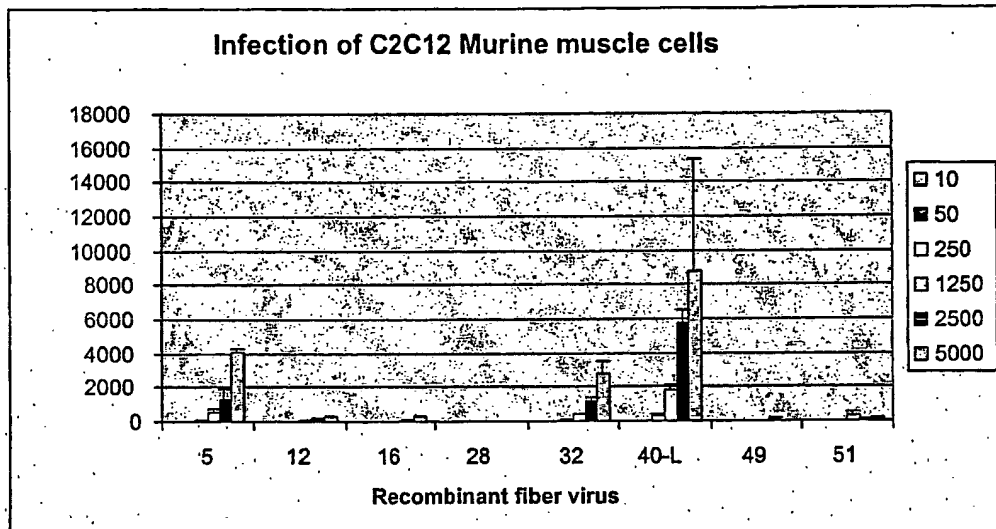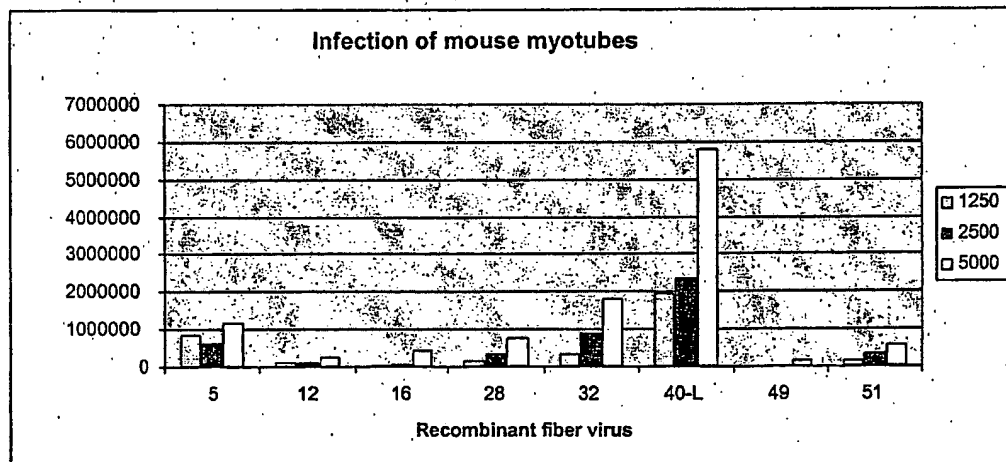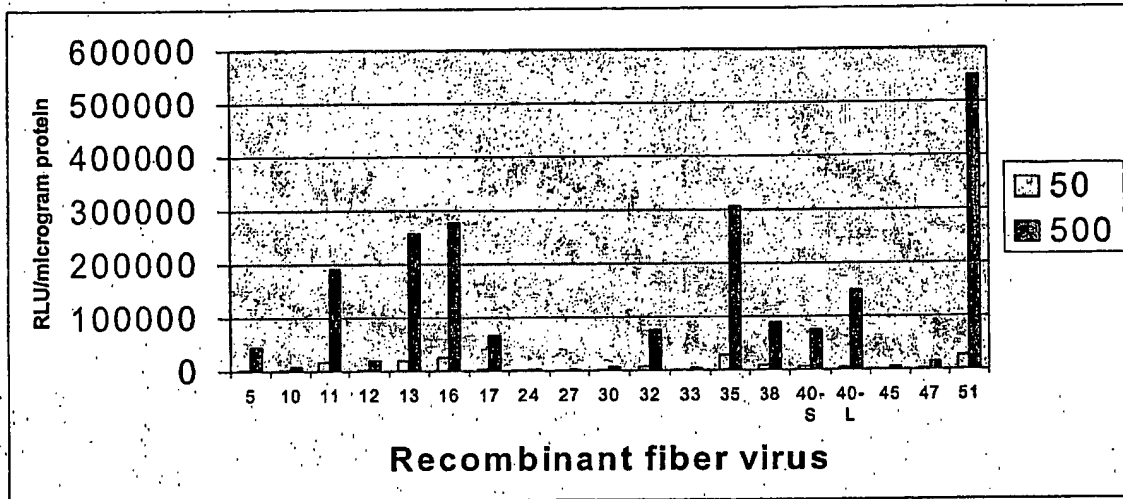   vehicle according to any one of claims 9-17.
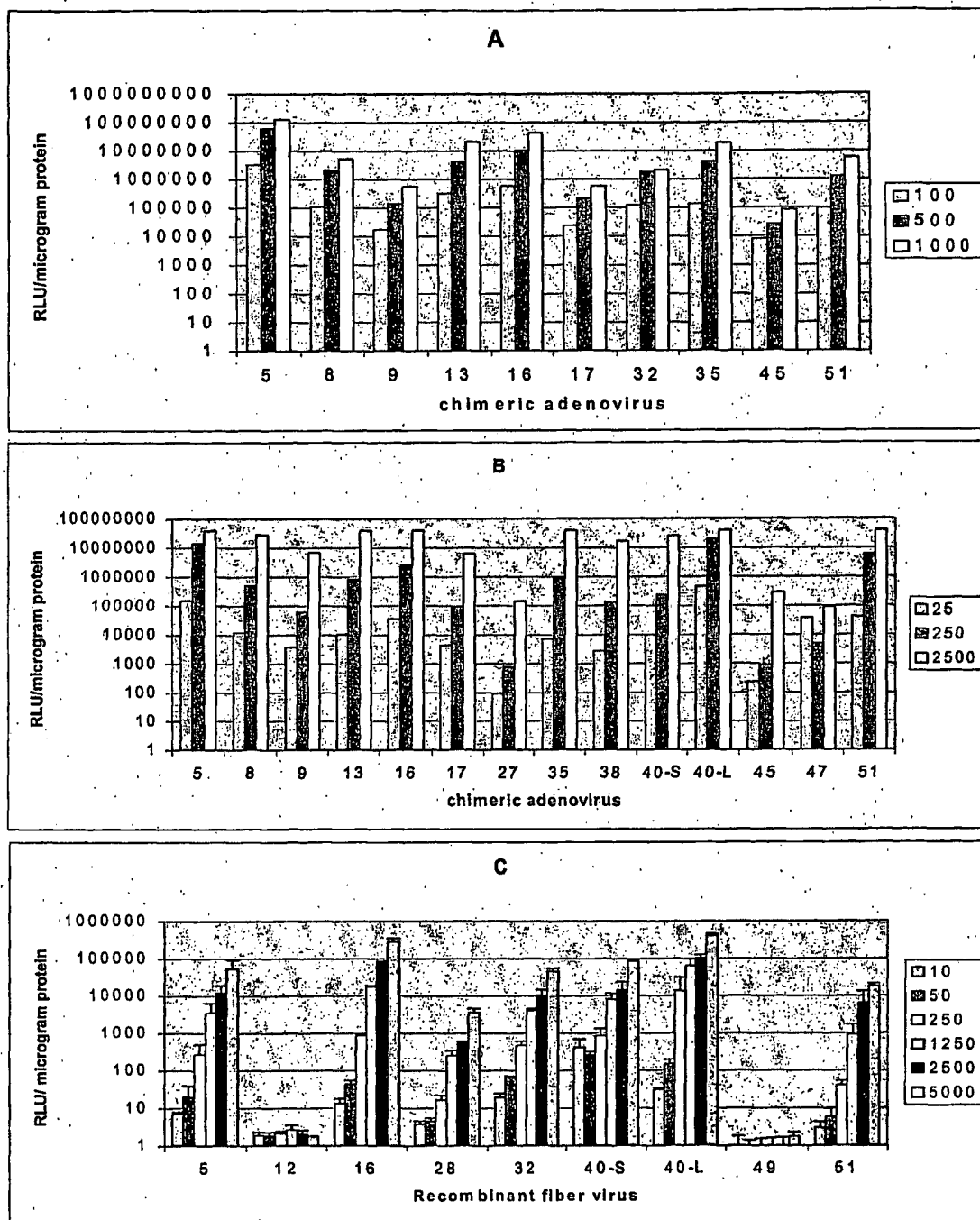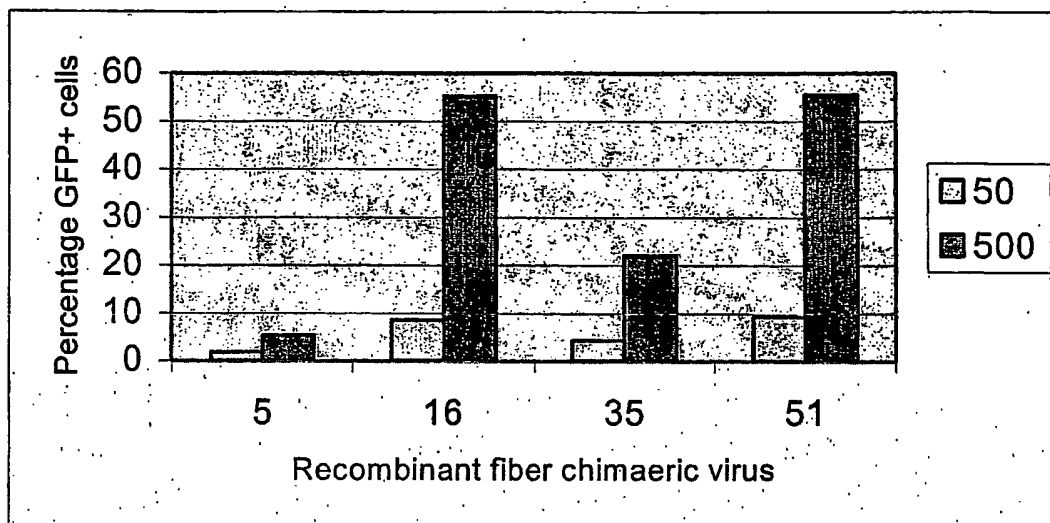
Figure 1

Figure 2a

Figure 2b

Figure 3

Figure 4a

Figure 4b
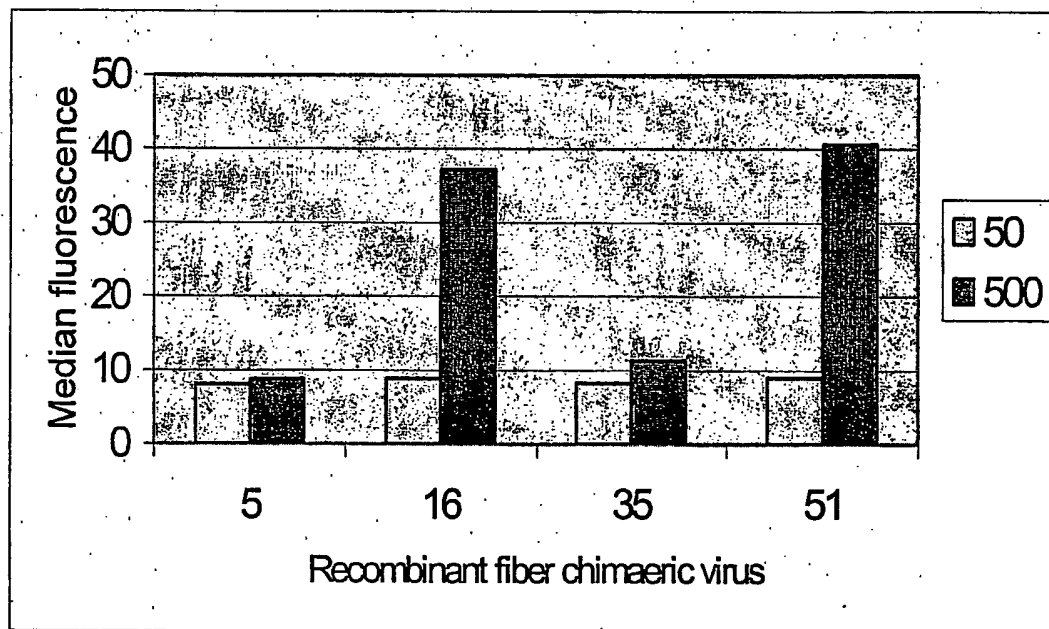
Figure 5
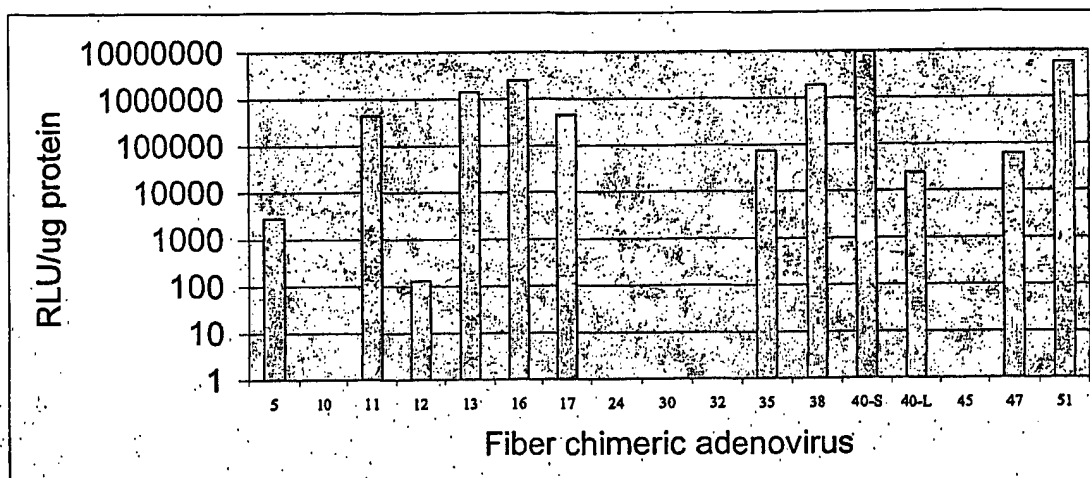
Figure 6
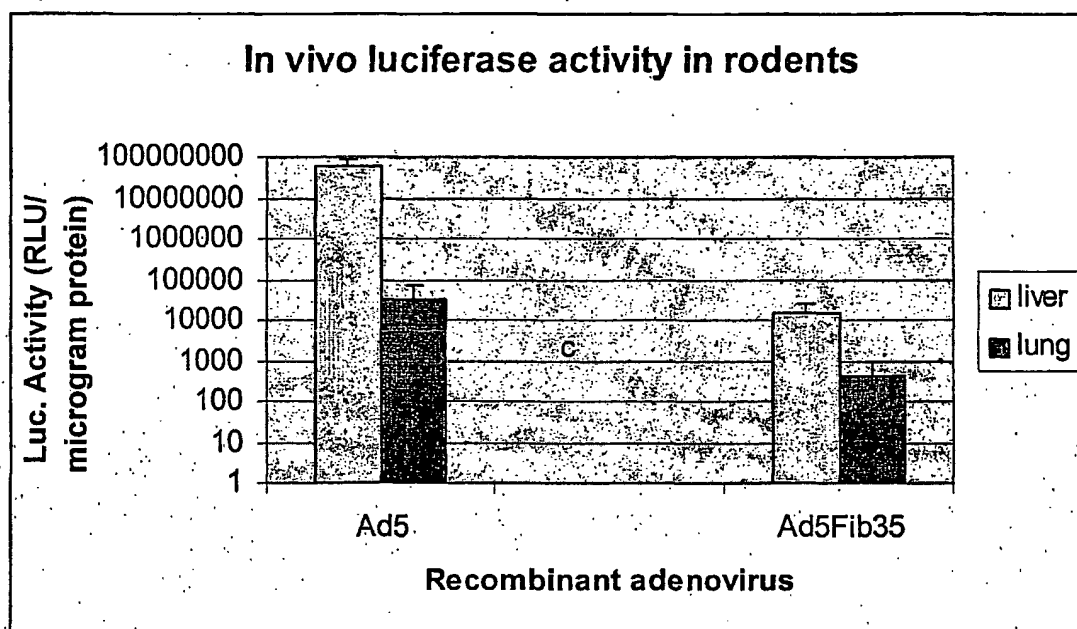
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   C12N15/86   C07K14/075   A61K38/00   A61K48/00   C12N15/00
        C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C07K   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 020 529 A (INTROGENE BV) 19 July 2000 (2000-07-19) the whole document ---	1-39
Y	US 5 871 727 A (CURIEL DAVID T) 16 February 1999 (1999-02-16) the whole document --- -/--	1-39

| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

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Date of the actual completion of the international search	Date of mailing of the international search report
11 December 2001	02/01/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fotaki, M

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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DOUGLAS J T ET AL: "Strategies to accomplish targeted gene delivery to muscle cells employing tropism-modified adenoviral vectors" NEUROMUSCULAR DISORDERS, PERGAMON PRESS, GB, vol. 7, July 1997 (1997-07), pages 284-298, XP002079944 ISSN: 0960-8966	1-5
Y	the whole document	9-14, 18-39
X	RAGOT T ET AL: "Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 361, no. 6413, 1993, pages 647-650, XP002162515 ISSN: 0028-0836 the whole document	9-12, 23-29, 34-38
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